EXHIBIT J

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Metabolic activation of *N*-alkylnitrosamines in genetically engineered *Salmonella typhimurium* expressing CYP2E1 or CYP2A6 together with human NADPH-cytochrome P450 reductase

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A *Salmonella typhimurium* tester strain YG7108 2E1/OR co-expressing human CYP2E1 together with human NADPH-cytochrome P450 reductase (OR) was established. The mutagen-activating capacity of human CYP2E1 for *N*-alkylnitrosamines was compared with that of CYP2A6 using the YG7108 2E1/OR and the YG7108 2A6/OR strains of *Salmonella*. *Salmonella* YG7108 2A6/OR is a derivative of YG7108 co-expressing CYP2A6 together with OR. Eight *N*-alkylnitrosamines, including *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodipropylamine (NDPA), *N*-nitrosodibutylamine (NDBA), *N*-nitrosomethylphenylamine (NMPhA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were examined. CYP2E1 expressed in the YG7108 2E1/OR cells showed mutagen-activating capacity, as indicated by induced revertants/min/pmol cytochrome P450, for NDMA, NDEA, NDPA, NDBA, NPYR and NNK, but not NMPhA and NNN. CYP2A6 activated NDMA, NDEA, NDPA, NDBA, NMPhA, NPYR, NNN and NNK. The ratio of the mutagen-activating capacity seen with CYP2A6 to that seen with CYP2E1 was calculated for each *N*-alkylnitrosamine. In the case of NDMA, NPYR and NDEA, the ratio was under 1.0, while the ratio was over 1.0 with NDPA, NDBA, NNK, NMPhA and NNN. We conclude that human CYP2E1 is mainly responsible for the metabolic activation of *N*-nitrosamines with a relatively short alkyl chain(s), whereas CYP2A6 was predominantly responsible for the metabolic activation of *N*-alkylnitrosamines possessing a relatively bulky alkyl chain(s).

Introduction

N-Alkylnitrosamines are recognized as one of the most potent chemical carcinogens present widely in the environment, as evidenced by the fact that they induce cancer in various organs in experimental animals, and maybe even in humans (1–5). The *N*-alkylnitrosamines require metabolic activation to exert their genotoxicity. The first activation step of *N*-alkylnitrosamines is thought to be hydroxylation of the carbon atom located at the α position of the *N*-nitroso group. The reaction is mainly catalyzed by cytochrome P450 (2,5). The subfamilies of cytochromes P450 mainly involved in hydroxylation of *N*-nitrosamines are thought to be CYP2E and CYP2A in humans (6–8).

The CYP2E subfamily contains two forms, CYP2E1 and CYP2E2 (9–11). Human CYP2E1 metabolizes drugs such as chlorzoxazone (12) and is responsible for the metabolic activation of *N*-nitrosodialkylamines such as *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) (6). Recently, the metabolism of *N*-nitrosodialkylamines such as NDMA, NDEA, *N*-nitrosodipropylamine (NDPA), *N*-nitrosodibutylamine (NDBA) and *N*-nitrosodiamylamine (NDAA) was investigated using human liver microsomes and cDNA-expressed human cytochromes P450 (13). The results showed that NDMA was metabolized primarily by human CYP2E1.

The CYP2A subfamily consists of at least 12 distinct forms (14). The forms in the CYP2A subfamily efficiently catalyze coumarin 7-hydroxylation (15–18). In humans, CYP2A6 is one member of the CYP2A subfamily. CYP2A6 is responsible for the metabolic activation of *N*-alkylnitrosamines including NDMA, NDEA, *N*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific nitrosamine (6,19). Recently, we successfully established a *Salmonella typhimurium* strain YG7108 2A6/OR co-expressing CYP2A6 and human NADPH-cytochrome P450 reductase (OR). The mutagen-activating capacity of CYP2A6 for various *N*-alkylnitrosamines was determined with genetically engineered bacterial cells. It was demonstrated that CYP2A6 was responsible for the metabolic activation of *N*-nitrosomethylphenylamine (NMPhA). To our knowledge, mutagenicity of NMPhA could not be detected by the Ames test using a parental YG7108 strain in the presence of S9 (9000 *g* supernatant fraction of liver homogenates) prepared from rat liver.

Despite these studies, no comparative studies have been reported so far to clarify the roles of human CYP2E1 and CYP2A6 in the metabolic activation of a series of *N*-alkylnitrosamines. Thus, in the present study, we first established a new *S.typhimurium* tester strain YG7108 2E1/OR co-expressing human CYP2E1 and human OR. We report herein a comparison of the capacity of human CYP2E1 with that of CYP2A6 to metabolically activate eight alkyl derivatives of *N*-nitrosamines using the YG7108 2E1/OR and YG7108 2A6/OR strains of *Salmonella*.

Abbreviations: δ-ALA, δ-aminolevulinic acid; G-6-P, glucose 6-phosphate; G-6-PDH, glucose 6-phosphate dehydrogenase; IPTG, isopropyl β-D(–)-thiogalactopyranoside; MC, minimal concentration; NDAA, *N*-nitrosodiamylamine; NDBA, *N*-nitrosodibutylamine; NDEA, *N*-nitrosodiethylamine; NDMA, *N*-nitrosodimethylamine; NDPA, *N*-nitrosodipropylamine; NMPhA, *N*-nitrosomethylphenylamine; NNK, 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone; NNN, *N*-nitrosonornicotine; NPYR, *N*-nitrosopyrrolidine; OR, NADPH-cytochrome P450 reductase.

Materials and methods

Chemicals

NDMA, NDEA, isopropyl β-D(–)-thiogalactopyranoside (IPTG), 4-nitrophenol, coumarin and cytochrome c were purchased from Wako Pure Chemical

Table I. *Salmonella typhimurium* strains and plasmids established or used in the present study[a]

Strain/plasmid	Character
Salmonella typhimurium strains	
L55000	*metA22 metE551, trpC2, ilv-452*, H2-e,n,x, *fla-66, rpsL120, xyl-404, leu, hsdL6, hsdSA29, hsdSB*
YG7108	*hisG46, gal*, Δ (*chl, uvrB, bio*), *rfa*, Δ ada_{ST}; Km^r, Δ ogt_{ST}; Cm^r
YG7108 pCW	YG7108 harboring pCW; Amp^r, Cm^r, Km^r
YG7108 2E1/OR	YG7108 harboring pCYP2E1/OR; Amp^r, Cm^r, Km^r
YG7108 2A6/OR	YG7108 harboring pCYP2A6/OR; Amp^r, Cm^r, Km^r
Plasmids	
pCW	Expression plasmid used for human CYP cDNA with human OR cDNA; Amp^r
pCYP2E1/OR	pCW carrying human CYP2E1 cDNA with modified N-terminus and human OR cDNA; Amp^r
pCYP2A6/OR	pCW carrying human CYP2A6 cDNA with modified N-terminus and human OR cDNA; Amp^r

[a]Amp^r, ampicillin resistance gene; Cm^r, chloramphenicol resistance gene; Km^r, kanamycin resistance gene.

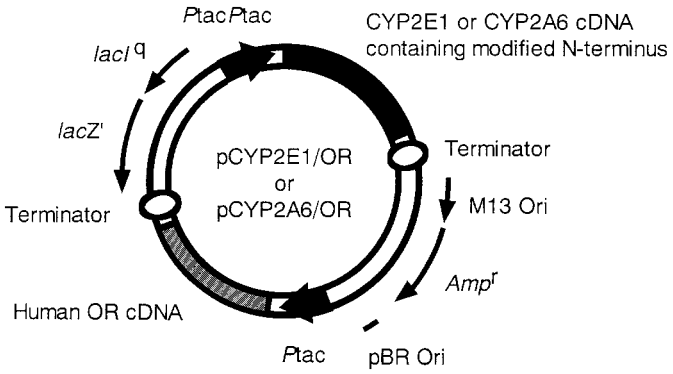

Fig. 1. Structure of a plasmid to co-express cytochrome P450 and OR in *S.typhimurium* YG7108 cells. The 9.3 kb plasmid contains cDNAs for cytochrome P450 and OR, which are linked to the *tac* promoter and terminator, respectively.

Industries (Osaka, Japan). NNN and NNK were obtained from Toronto Research Chemicals (Toronto, Canada). Glucose 6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-PDH) and $NADP^+$ were from Oriental Yeast (Tokyo, Japan). NDPA, NDBA, NMPhA, *N*-nitrosopyrrolidine (NPYR) and δ-aminolevulinic acid (δ-ALA) were from Tokyo Chemical Industry (Tokyo, Japan).

Salmonella typhimurium tester strains and plasmids

Salmonella typhimurium tester strains and plasmids used in this study are summarized in Table I. YG7108, derived from TA1535, lacks two O^6-methylguanine-DNA methyltransferase genes, *ada* and *ogt* (20). An expression plasmid pCYP2E1/OR (Figure 1) carrying CYP2E1 and human OR cDNAs (21) was first modified by introduction into *S.typhimurium* strain LB5000 (R^-M^+) (22) and then the plasmid was introduced into YG7108 cells.

Culture conditions for expression of CYP2E1 or CYP2A6 with OR in S.typhimurium YG7108

Expression of CYP2E1 or CYP2A6 with OR in the *Salmonella* cells was achieved as follows. Twenty microliters of a bacterial stock solution were inoculated into 10 ml of Luria–Bertani medium supplemented with ampicillin (100 mg/ml), kanamycin (25 mg/ml) and chloramphenicol (10 mg/ml). The bacteria were grown with shaking at 37°C for 12 h. Two milliliters of the culture were inoculated into 200 ml of a modified Terrific Broth (23) and the bacteria were grown with shaking at 37°C for 2 h prior to induction with 1.5 mM IPTG. The expression of recombinant proteins was achieved by a further incubation at 30°C for 18 h with shaking.

Assay of catalytic activity

All assays were carried out with whole bacterial cells expressing CYP2E1 or CYP2A6 with OR. The amount of the bacterial preparation added to the incubation mixture will be shown as the amount of cytochrome P450. All reactions were initiated by addition of substrate.

4-Nitrophenol hydroxylase activity of CYP2E1 expressed in the bacterial cells was assayed essentially as described by Tassaneeyakul *et al*. (25). Briefly, the incubation mixture contained 100 mM potassium phosphate buffer (pH 6.8), 200 μM 4-nitrophenol and 3.3 pmol of CYP2E1 in a final volume of 0.5 ml. Incubations were carried out at 37°C for 60 min. The metabolite was extracted with 4 ml of diethyl ether. Analysis of the metabolite, 4-nitrocatechol, was performed by HPLC (Shimadzu, Kyoto, Japan) on a Capcell Pak C18 column (4.6×250 nm, SG120A, 5 μm; Shiseido, Tokyo, Japan) with a SPD-6A UV absorbance detector (Shimadzu, Kyoto, Japan). The column temperature was 40°C. Metabolites were separated using a solvent system containing 22% acetonitrile, 1% acetic acid and 77% trimethylamine (30 mM, pH 3.0). The flow rate was 1.0 ml/min. The elution of 4-nitrophenol and its metabolites was monitored at 250 nm.

Coumarin 7-hydroxylase activity of CYP2A6 expressed in whole bacterial cells was measured essentially according to the method of Pearce *et al*. (24). Briefly, the incubation mixture consisted of 25 mM potassium phosphate buffer (pH 7.4), 50 μM coumarin and 10 pmol of CYP2A6 in a final volume of 1.0 ml. Incubations were carried out at 37°C for 15 min. The metabolite, 7-hydroxycoumarin, was determined fluorometrically.

Mutation assay

Mutation assays with YG7108 2E1/OR and YG7108 2A6/OR were carried out as follows. Cultures were in 200 ml of modified Terrific Broth (23) and were grown with shaking at 37°C for 2 h prior to induction with 1.5 mM IPTG. Expression of recombinant proteins was achieved by a further incubation at 30°C for 18 h with shaking. The number of cells was adjusted to give 1–2×10^9 cells/ml by dilution with modified Terrific Broth. The assay was carried out as described by Maron and Ames (26) with minor modifications. Bacterial cells were pre-exposed to an *N*-alkylnitrosamine at 37°C for 20 min before plating.

The plates were incubated at 37°C for 2 days. Assays were carried out in triplicate at each dose. The results were judged as positive when the number of colonies increased in a dose-dependent manner and reached a level twice as high as that obtained with vehicle alone as a control. The minimal concentration (MC) value of *N*-alkylnitrosamines was defined as the concentration of a promutagen giving a positive result.

Other methods

The content of cytochrome P450 in bacterial cells was determined by Fe^{2+}–CO versus Fe^{2+} difference spectra, according to the method of Omura and Sato (27). *Salmonella* cells were suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 0.2% (w/v) Emulgen 911. The difference spectra were recorded using a UV-visible spectrophotometer model MPS-2000 (Shimadzu, Kyoto, Japan). The OR activity in sonicated bacterial cells was measured with cytochrome *c* as the electron acceptor by detecting the absorbance change at 550 nm at 20°C according to the method of Phillips and Langdon (28). Protein concentrations were determined as described by Lowry *et al.* (29).

Results

Expression of CYP2E1 or CYP2A6 together with OR in S.typhimurium YG7108

An expression plasmid pCYP2E1/OR carrying CYP2E1 cDNA together with OR cDNA was introduced into *S.typhimurium* YG7108, which shows an *ada*- and *ogt*-deficient genotype. The expression of CYP2E1 holoprotein in YG7108 2E1/OR was determined with Fe^{2+}–CO versus Fe^{2+} difference spectra (Figure 2). The expression level of CYP2E1 in comparison with that of CYP2A6 and the level of OR in bacterial cells are summarized in Table II. The expression levels of CYP2E1 and CYP2A6 were 15 ± 3 and 77 ± 8 nmol/l culture,

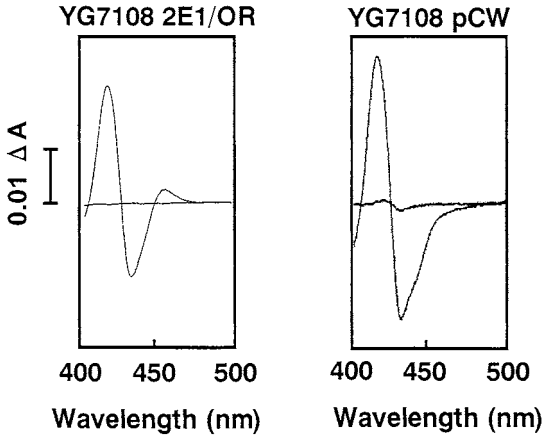

Fig. 2. Typical CO difference spectra of CYP2E1 and CYP2A6 expressed in established *S.typhimurium*. The content of CYP2E1 or CYP2A6 in *S.typhimurium* cells was determined by Fe^{2+}–CO versus Fe^{2+} difference spectra.

Table II. Expression levels of cytochrome P450 and OR in S.*typhimurium* YG7108 cells co-expressing CYP2A6 or CYP2E1 and OR

Strain	Cytochrome P450 content[a] (nmol/l culture)	OR activity (μmol cytochrome *c* reduced/min/l culture)
YG7108 2E1/OR	15 ± 3	390 ± 17
YG7108 2A6/OR	77 ± 8	470 ± 20
YG7108 pCW	<5	5 ± 2

Values are presented as means ± SD (n = 3).
[a]CYP2E1 or CYP2A6 content was determined by Fe^{2+}–CO versus Fe^{2+} difference spectra.

Table III. Catalytic activities of CYP2E1 and CYP2A6 in *S.typhimurium* YG7108 cells

Strain	4-Nitrophenol hydroxylation (nmol/min/nmol cytochrome P450)	Coumarin 7-hydroxylation (nmol/min/nmol cytochrome P450)
YG7108 2A6/OR	nd	16.3 ± 1.8
YG7108 2E1/OR	19.5 ± 2.2	nd
YG7108 pCW	<0.1	<0.1

Specific values are presented as means ± SD (n = 3).
nd, not determined.

respectively. No detectable cytochrome P450 was seen in the YG7108 pCW cells.

The expression levels of OR were 390 ± 17 and 470 ± 20 μmol cytochrome c reduced/l culture for YG7108 2E1/OR and YG7108 2A6/OR, respectively. Only a small amount of OR activity was detectable in strain YG7108 pCW.

Enzyme activities in established S.typhimurium whole cells

The catalytic activities of CYP2E1 and CYP2A6 expressed in YG7108 2E1/OR and YG7108 2A6/OR cells were determined using as substrates 4-nitrophenol for CYP2E1 and coumarin for CYP2A6 (Table III). The catalytic activities of CYP2E1 and CYP2A6 for 4-nitrophenol hydroxylation and coumarin 7-hydroxylation were 19.5 ± 2.2 and 16.3 ± 1.8 nmol/min/nmol cytochrome P450, respectively. Activity was not detectable in the parental YG7108 pCW cells.

Mutation assay with YG7108 2E1/OR and YG7108 2A6/OR cells for N-alkylnitrosamines

The mutagen-producing activity of CYP2E1 for *N*-alkylnitrosamines was compared with that of CYP2A6 using established *S.typhimurium* YG7108 2E1/OR and YG7108 2A6/OR, respectively. Eight *N*-alkylnitrosamines, NDMA, NDEA, NDPA, NDBA, NMPhA, NPYR, NNK and NNK, were employed as promutagens. The results are shown in Figure 3. CYP2E1 expressed in the genetically engineered *S.typhimurium* cells activated NDMA, NDEA, NDPA, NDBA, NPYR and NNK, but not NMPhA and NNN, while CYP2A6 was capable of activating all promutagens tested. The MC values defined as described in Materials and methods for YG7108 2E1/OR ranged from 1.43 μM for NDPA to 1.43 mM for NDMA. The MC values for YG7108 2A6/OR ranged from 0.143 μM for NMPhA to 1.43 mM for NDMA (Table IV).

The capacity of CYP2E1 and CYP2A6 to activate *N*-alkylnitrosamines is summarized in Table V. CYP2E1 activated *N*-alkylnitrosamines with a capacity ranging from 12.2 to 476 induced revertants/min/pmol cytochrome P450/nmol mutagen for NNK and NDEA, respectively. CYP2A6 activated *N*-alkylnitrosamines with a capacity ranging from 0.322 to 7590 induced revertants/min/pmol cytochrome P450/nmol mutagen for NDMA and NMPhA, respectively. The ratios of the values with CYP2A6 to those with CYP2E1 are also shown in Table V. In the case of NDMA, NPYR and NDEA, the ratio was below 1.0, indicating that CYP2E1 activates these promutagens more efficiently than does CYP2A6. On the other hand, the ratio was over 1.0 with NDPA, NDBA, NNK, NMPhA and NNN. These results indicate that CYP2E1 is mainly responsible for the metabolic activation of *N*-alkylnitrosamines with relatively short alkyl chain(s), whereas CYP2A6 is predominantly responsible for the metabolic activation of *N*-alkylnitrosamines possessing a relatively bulky alkyl chain(s).

Discussion

N-alkylnitrosamines cause cancers in many organs of experimental animals and are considered as one of the most important chemical carcinogens in the environment (1–5). The metabolic activation of *N*-alkylnitrosamines by cytochrome P450 enzymes may be critical in the initiation of human cancer (32). CYP2E1 and CYP2A6 expressed in human liver microsomes have been reported to be the major enzymes in the metabolic activation of *N*-alkylnitrosamines such as NDMA, NDEA, NNK and NNN (6–8).

To clarify the roles of CYP2E1 and CYP2A6 in the metabolic activation of *N*-alkylnitrosamines in detail, we established *Salmonella* YG7108 cells expressing CYP2E1 or CYP2A6 together with OR (YG7108 2E1/OR and YG7108 2A6/OR). CYP2E1 and CYP2A6 were sufficiently expressed in the genetically engineered *Salmonella* cells. In the case of CYP2E1, the level of CYP2E1 expressed in 1 l of the culture of YG7108 2E1/OR cells corresponded to that expressed in 23 g of human liver, assuming that 1 g of human adult liver contained ~0.66 nmol of CYP2E6 protein, referring to the data reported by Shimada *et al*. (30). The expression level of CYP2A6 in 1 l of a culture of YG7108 2A6/OR cells corresponded to that expressed in 193 g of human liver, assuming that 1 g of human adult liver contained ~0.4 nmol of CYP2A6 protein.

The endogenous electron transport system of *Salmonella* YG7108 cells is able to support only a small portion of the

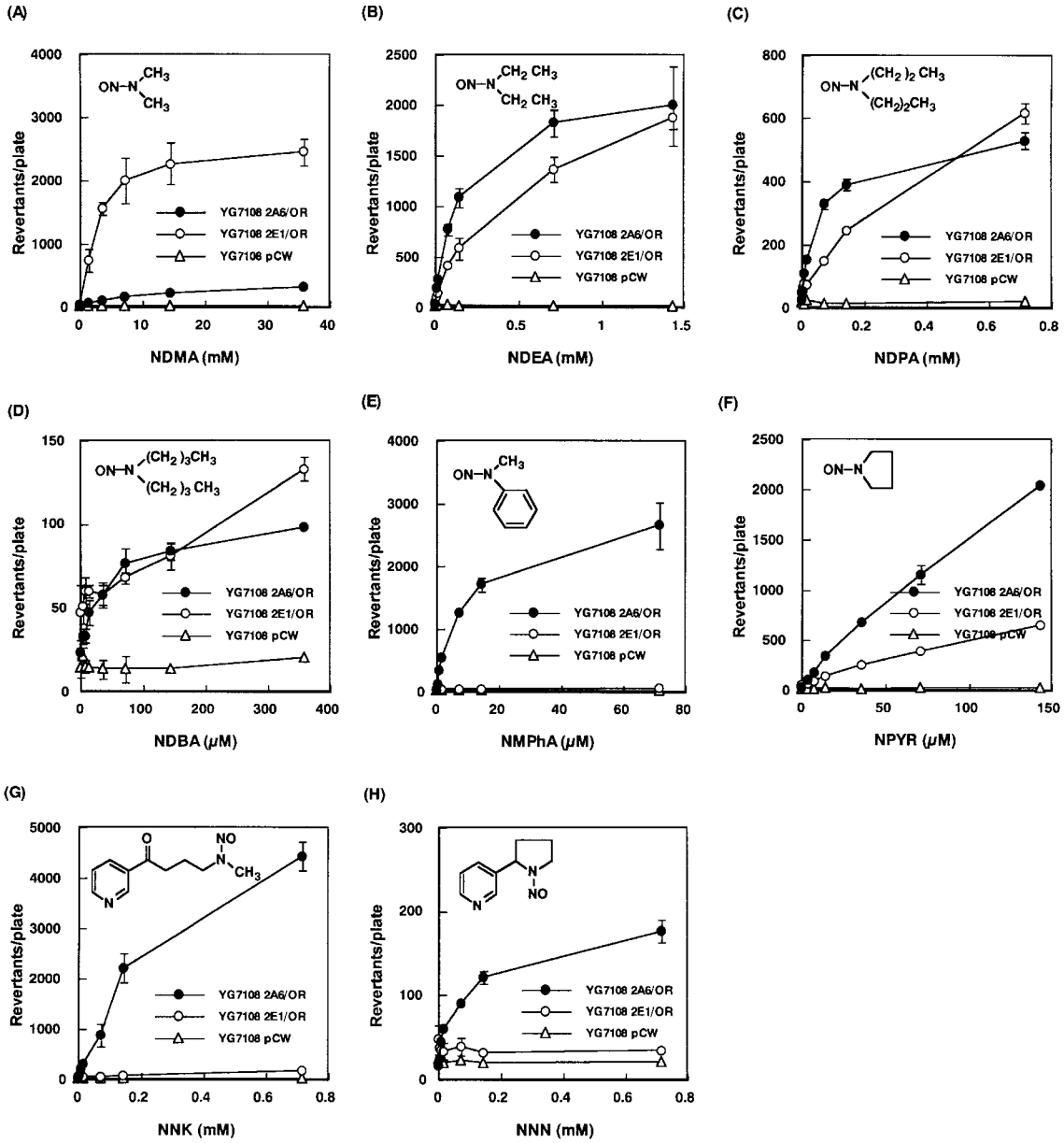

Fig. 3. Comparison of the mutagen-activating capacity of CYP2E1 and CYP2A6 for various *N*-alkylnitrosamines. *Salmonella typhimurium* expressing CYP2E1 or CYP2A6 was treated with NDMA (**A**), NDEA (**B**), NDPA (**C**), NDBA (**D**), NMPhA (**E**), NPYR (**F**), NNK (**G**) and NNN (**H**) at 37°C for 20 min. Immediately after preincubation, the reaction mixture was poured onto a minimal glucose plate with top agar. The plates were incubated at 37°C for 2 days before the number of His^+ revertants per plate was counted. Data points are shown as the mean (n = 3). Bars indicate standard deviation.

cytochrome P450 activity (33). Thus, OR, which is capable of transferring electrons from NADPH to cytochrome P450, was co-expressed in the *Salmonella* cells with cytochrome P450 in order to obtain full catalytic activity of cytochrome P450. As a result, CYP2E1 and CYP2A6 expressed in the *Salmonella* cells efficiently catalyzed 4-nitrophenol hydroxylation and coumarin 7-hydroxylation, respectively (Table III).

Reactive intermediates produced by cytochrome P450 are, in general, believed to be chemically unstable and reactive with bacterial cell components. Therefore, as is the case with the classical Ames test, the reactive metabolites are assumed to bind to components on the cell surface if the reactive intermediates are formed outside the cell by drug-metabolizing enzymes contained in S9 mix. Expression of cytochrome P450 in the *Salmonella* cells is expected to lead to intracellular production of reactive intermediates, resulting in efficient binding of reactive metabolites to DNA, causing mutations in the *Salmonella* genome. YG7108 cells lack two O^6-methylguanine-DNA methyltransferase genes, *ada* and *ogt*. Since *N*-alkylnitrosamines induce DNA mutations via alkylation of purines and pyrimidines (34), the properties of the established *Salmonella* strain might also make it highly sensitive to *N*-alkylnitrosamines.

The capacity for mutagenic activation of *N*-alkylnitrosamines was not affected by the addition of NADPH, suggesting that *Salmonella* could generate sufficient NADPH for cytochrome P450 activity.

We have shown that the contribution of CYP2E1 was larger than that of CYP2A6 in the activation of NDEA. However, previous reports demonstrated a high correlation between coumarin 7-hydroxylase activity and the formation of acetaldehyde from NDEA and a poor correlation between CYP2E1

Table IV. Comparison of the MC values of CYP2E1 with those of CYP2A6 with different *N*-alkylnitrosamines

N-alkylnitrosamine	MC value[a]	
	YG7108 2E1/OR	YG7108 2A6/OR
NDMA	1.43 mM	1.43 mM
NDEA	7.14 μM	7.14 μM
NDPA	1.43 μM	1.43 μM
NDBA	0.357 mM	14.3 μM
NMPhA	nd[b]	0.143 μM
NPYR	14.3 μM	3.57 μM
NNK	0.714 mM	1.43 μM
NNN	nd	7.14 μM

[a]The MC value of a *N*-alkylnitrosamine is defined as the concentration of a promutagen giving a positive result.
[b]Not detectable, the number of colonies did not reach a level twice as high as that obtained with vehicle alone as a control.

Table V. Comparison of the mutagen-activating capacity of CYP2E1 with that of CYP2A6 with *N*-alkylnitrosamines

N-alkylnitrosamine	Mutagen-activating capacity[a]/nmol mutagen		Ratio B:A
	YG7108 2E1/OR (A)	YG7108 2A6/OR (B)	
NDMA	31.8	0.322	0.0101
NDEA	476	326	0.685
NDPA	249	279	1.12
NDBA	15.6	23.4	1.50
NMPhA	nd	7590	∞
NPYR	409	240	0.587
NNK	12.2	625	51.2
NNN	nd	68.7	∞

nd, not detectable, the number of colonies did not reach a level twice as high as that obtained with vehicle alone as a control.
[a]Induced revertants/min pre-exposure/pmol cytochrome P450.

content and acetaldehyde formation from NDEA (13,35). Thus, the previous data indicated that CYP2A6 was involved in the metabolic activation of NDEA, while CYP2E1 was not. The apparent discrepancy between the previous and the present data may be accounted for by the fact that the previous investigators measured acetaldehyde formation from NDEA at high concentrations of NDEA (2.00–4.00 mM), because of the low sensitivity of the method (36) to detect aldehyde formation, while we carried out experiments with NDEA at a low concentration (7.14 μM).

Bellec *et al*. (13) examined aldehyde formation via α-hydroxylation using symmetrical *N*-dialkylnitrosamines such as NDMA, NDEA, NDPA, NDBA and NDAA. They demonstrated that CYP2E1 was responsible for α-hydroxylation of *N*-alkylnitrosamines with short alkyl chains, measuring aldehyde formation from the *N*-alkylnitrosamines. The same tendency was found in the present study on the role of CYP2E1 in the mutagenic activation of these chemicals as that seen in the previous study. In the present study we also examined the role of CYP2E1 in the metabolic activation of *N*-alkylnitrosamines with non-symmetrical alkyl chains, such as NNK and NMPhA, in addition to *N*-alkylnitrosamines with symmetrical alkyl chains. Although NNK and NMPhA possess a methyl group at the α position of the carbon atom bonded to the nitroso group, these were not efficiently activated by CYP2E1. These results suggest that the methyl group at the α position of the carbon atom bonded to the nitroso group may not be a substrate determinant for CYP2E1 and that molecular mass may be the main determinant of a CYP2E1 substrate. On the other hand, we have demonstrated that CYP2A6 is involved in the metabolic activation of all the *N*-alkylnitrosamines examined, while CYP2E1 was not responsible for metabolic activation of NMPhA and NNN (Figure 3 and Table V). The results suggest that CYP2A6 has a wider substrate specificity for *N*-alkylnitrosamines than does CYP2E1. The metabolic activation of many other *N*-alkylnitrosamines should be investigated to prove this hypothesis.

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